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Serodiagnosis of New Viral Disease of Sunflower in Tamil Nadu

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Sunflower (*Helianthus annuus* L.) cultivation has been hampered by a new viral disease in Tamil Nadu. The coat protein size of the purified virus was determined as 29 kDa in SDS-PAGE. Purified preparation of virus under transmission Electron Microscope revealed the presence of isometric particles. In DAC-ELISA test, plants with different symptoms of SFNV were found to be positive when reacting with the homologous antiserum but not with the heterologous antiserum raised against GBNV. Concentration of the virus was found to be more in the stem portion that recorded the absorbance value of 0.893 which was followed by leaf (0.719), petiole (0.501) and calyx (0.419) whereas it was 0.072 in buffer control. Concentration of the virus also got increased with increase in days after inoculation. An absorbance value of 0.523 recorded 10 days after inoculation was increased to 1.060 in 21 days after inoculation.

Key words: Sunflower, necrosis virus, serology, ELISA, ISEM

Sunflower (*Helianthus annuus* L.) is the third important edible oilseed crop in the world after soybean and groundnut. It is being hampered by the occurrence of a new necrosis disease. This disease was noticed for the first time in the country during 1997 at Bagepally of Kolar district and around Bangalore of Karnataka in seed production plots and caused panic among farmers and seed growers (Nagaraju and Hanumantha Rao,1999).

The symptoms of the disease appear as necrosis (death of the tissue) on one part of leaf lamina near the midrib resulting in twisting of the leaf. Later, the necrosis extends through one side of the lamina to the petiole and stem and finally terminate at shoot of the plant. The tip of the growing plants become necrotic giving typical necrosis symptoms. The plants fail to produce flowers if infected early and finally die (Nagaraju and Hanumantha Rao, 1999). In order to make detailed investigation, the present study was contemplated to characterize the virus using serological assays.

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Materials and Methods

Purification of virus

The virus infecting sunflower was purified by the method adopted by Salazer *et al.* (1981) and Ramiah *et al.* (2001a) with some modifications in the incubation period and speed of centrifugation. An enamel tray was filled with tap water to three fourth of the capacity. A pair of sterilized pestle and mortar was kept in the tray that was then placed in the freezer till the water was frozen. Sodium phosphate buffer of 0.1 M (pH 7.2) containing 0.1 per cent 2- mercaptoethanol was prepared and stored at 4°C. Plant parts showing characteristic symptoms were homogenized separately with the addition of one ml of 0.1 M sodium phosphate buffer for every gram of plant material in the mortar kept in the tray.

The homogenate was squeezed through muslin cloth and the sap was used as standard inoculum for inoculation. Cowpea plants of cv. C 152 were used for propagating the virus isolates since they produce typical local lesions within 3-4 days after inoculation.The primary

leaves of cowpea plants (cv. C.152) showing chlorotic spots, necrotic spots and veinal necrosis were harvested and triturated at 1:2 dilution (W/V) in 0.06 M sodium phosphate buffer (pH 7.2) containing 0.1 per cent 2-mercaptoethanol and 0.001 M EDTA. The sap was emulsified with 1/5th volume of cold chloroform by stirring continuously for 15 min. and centrifuged at 10,000 g for 15 min. The buffer layer was removed and the virus was precipitated by adding polyethylene glycol (PEG. 6000, 6% w/v) in the presence of sodium chloride (1.75% w/v) with continuous stirring for 20 minutes.

Then the mixture was allowed for an incubation period of about 2.5 h. Again the virus was sedimented by centrifugation at 10000 g for 30 min. The sediment was suspended in 1/5th volume of sodium phosphate buffer (0.06 M, pH 7.2) and stirred for 2 h. Virus suspension was further clarified by two cycles of differential centrifugation at 10,000 g for 10 min. followed by 29,000 rpm for 2.5 h. Virus pellet thus obtained was dissolved in 3 ml of 0.06 M sodium phosphate buffer (pH 7.2) and was layered on a linear 100 to 400 mg/ml preformed sucrose density gradient (prepared by layering 5 ml of 100 mg/ml and 10 ml each of 200, 300 and 400 mg/ml sucrose in 0.06 M sodium phosphate buffer (pH 7.2). Then the gradient solution was incubated at 4°C overnight. Next day, the gradients were centrifuged at 20,000 rpm for 3 h and observed for the appearance of light scattering zone that represents the presence of virus particles in the particular zone.

Analysis of nucleocapsid protein

Virus nucleocapsid protein was analysed by SDS-PAGE as described by Laemmli (1970). Gel (1mm thickness) was casted in a gel mold, consisting of two glass plates, spacers and well forming comb. Two sides of the gel mold were closed by placing two spacers on either side. The bottom edge was sealed with molten agar (3%) by keeping the mold in a gel boat. The resolving gel solution was prepared and pipetted immediately into the gel mold gently from the sides. Distilled water was overlaid on the top of the resolving gel. After polymerization, the water layer was decanted carefully. Freshly prepared stacking gel was poured then the comb was inserted into the stacking gel and allowed for polymerization at room temperature for 30 min. The comb was removed gently from the gel mold, wells were marked and clamped to the electrophoresis unit.

Raising of polyclonal antisera

Purified virus sample (500 ml) emulsified with Freund's complete adjuvant (1:1 v/v) was injected intramuscularly into the thigh muscle of New zealand white rabbit. After one week, next injection of the purified virus but with Freund's incomplete adjuvant was given to the opposite flank of the animal to that previously used. The injection with Freund's incomplete adjuvant was repeated with weekly interval to complete five injections. Two weeks after the last injection, the fur from the region over the central ear artery or marginal ear vein was removed using a new scalpel or razor blade. A drop of ethanol was applied over the shaved area to stimulate the supply of blood. On the marginal ear vein a cut was made using a razor blade and the blood was collected in a glass container. Firm pressure was given to the artery until the bleeding stops. The blood was allowed to clot at room temperature for 1-2 h at slanting position and then at 4° C overnight in the same position. The supernatant serum having the yellow colour was collected in Eppendorf tubes. To make the serum free from any blood cell the serum was subjected to centrifugation at 5000 rpm for 10-15 min. At weekly intervals, the bleeding procedure was repeated four times. Then part of the antiserum was mixed with 50 per cent glycerol and stored at -20°C. Remaining part of the antiserum was added with 0.02 per cent sodium azide and stored at 4°C (Kannan *et al.,* 2003).

Serology

Direct antigen coating ELISA (DAC-ELISA)

The procedure adopted by Ramiah *et.al.* (2001a,b) was followed to test the samples under DAC-ELISA. Test/control samples (200 µl) extracted or diluted in coating buffer containing 2 per cent (w/v) PVP were added to each well

and the plate was covered with aluminium foil and incubated at 37° C for 30 min to 1 h. Then the plate was made empty and washed by flooding the wells with PBS-T (Phosphate Buffer saline-T)for 3 min. These washing and soaking operations were repeated thrice and the residual liquid was drained out on a paper towel. To the plate 200 ML of blocking solution (Bovine Serum Albumin 1%) was added and incubated for 1 h at 37°C. The plate was made empty and washed thrice with PBS-T. Primary antibody previously cross absorbed with the healthy sample was diluted up to 1: 2000 in PBS-TPO and added @ 200 µl / well and incubated for 2 h at 37° C or overnight at 4° C. Again the plate was washed thrice with PBS-T and to which the enzyme labelled (alkaline phosphatase, ALP) antirabbit IgG (secondary antibody) diluted up to 1:20000 in PBS-TPO was added @ 200 µl / well and incubated for 2 h at 37° C or overnight at 4° C. Then the substrate (Para nitrophenylphosphate, 0.5 to 1 mg/ml) was added @ 100 µl/well and incubated at room temperature for 1- 2 h or as long as necessary to observe the reaction. The reaction was then terminated by adding 50 ml of 3 M NaOH to each well. Then, the plate was assessed by visual observation or by measuring the absorbance at 405 nm in an ELISA reader.

Detection of SFNV in infected samples

The samples including different symptoms of sunflower necrosis disease (Complete stem necrosis, partial stem necrosis, top necrosis, yellow blotch, mild and severe necrosis) were tested for the presence of virus using DAC – ELISA. The polyclonal antiserum raised against SFNV, GBNV and TSV (groundnut) were used to detect the virus in infected samples. Healthy leaves of sunflower and cowpea served as control.

Concentration of virus in different parts of the plant

The distribution of SFNV in different parts of the plant viz., leaf, petiole, stem, calyx, pollen and seed were evaluated using DAC (Direct Antigen Coating) ELISA as per Ramiah et al., (2001 a,b)

Detection of SFNV through DAC-ELISA at different periods of inoculation

Twenty days old sunflower plants (cv.Co 4) were inoculated with SFNV and observed periodically upto 21 days for symptom development. The concentration of the virus at 3, 7, 10, 14, 18 and 21 days after inoculation was detected using DAC-ELISA.

Immunosorbent electron microscopy (ISEM)

A drop of crude antiserum (20 µl) was pipetted out on a parafilm membrane in moist petridish and a grid with coated side was floated on the drop. The petridish was incubated for a period of 30 min to 1 h at room temperature. Then the antiserum coated grid (ACG) was washed with phosphate buffer for 10 min. After that, a drop of extract from diseased tissue was pipetted out on a parafilm membrane and in the same way the grid was floated on it. Incubation was allowed for 30 min. to 2 h. Then the grid was washed with buffer for 10 min. Again the grid was placed on a drop of 1: 50 dilution of antiserum and incubated at 37° for 30 min. to 1 h. After the incubation, the grid was washed with water followed by 2 per cent aqueous uranyl acetate. Before going to observe under Electron microscope the grid was allowed for complete drying. Then the virus particles trapped and decorated by the antiserum were observed under EM (Ramiah *et.al.*2001a,b)

The particle size measurements were done as described by Thomas (1986). The diameter of the particles in millimeter was converted into equivalent value of nanometer. It was further divided by the corresponding magnification to get the size of a virus.

$$\text{Size of a virus particle (nm)} = \frac{\text{Diameter in mm} \times 10^6}{\text{Magnification}}$$

Results and Discussion

Purification of the virus

In the present study no virus band was observed as a light scattering zone in the sucrose gradient column. Ramiah *et al*. (2001 b) also

reported that SFNV as a band could not be located in the column as light scattering zone. Ladha lakshmi (2002) also got the same result when purified the TSV from blackgram. Hence, the column was fractionated using a hypodermic syringe into 12 fractions of 1 ml each starting from top of the tube and inoculated on cowpea to express the symptom. The fraction containing the virus particles was further centrifuged and from which the purified virus was separated.

By running SDS-PAGE, the coat protein size of virus was determined as 29 kDa. This is in agreement with Cook *et al*. (1999) who reported that tobacco streak virus infecting groundnut had migrated as a single band near 29 kDa in SDS-PAGE.

Table 1. Determination of titre of polyclonal antiserum raised against sunflower necrosis virus

Antigen dilution	Antibody dilution / Absorbance value at 405 nm *						
	1 : 50	1: 100	1: 200	1:500	1:1000	1: 2000	1: 5000
1:50	0.567	0.732	0.643	0.272	0.133	0.214	0.101
1:100	0.498	0.523	0.869	0.212	0.248	0.163	0.105
1:200	0.567	0.756	0.421	0.141	0.212	0.141	0.108
1:500	0.149	0.232	0.382	0.124	0.163	0.091	0.082
1:1000	0.101	0.212	0.321	0.102	0.099	0.024	0.090
Buffer	0.054	0.059	0.060	0.057	0.060	0.060	0.054

*Mean of three replications

Table 2. Detection of SFNV in infected samples using SFNV and GBNV antiserum

Sample No.	Description	*Absorbance at 405 nm**			
		SFNV		GBNV	
		After 1 h	After 2 h	After 1 h	After 2 h
1.	Complete necrosis on stem, leaves and petiole of sunflower	0.254+	0.588+	0.005 -	0.025
2.	Severe mosaic	0.028 +	0.102 +	0.001 -	0.019 -
3.	Top necrosis	0.234 +	0.574 +	0.001 -	0.026 -
4.	Yellow blotch	0.004 -	0.011 -	0.002 -	0.024 -
5.	Mild mosaic	0.001 -	0.061 +	0.002 -	0.015 -
6.	Mild mosaic + yellowing	0.040 +	0.150 +	0.005 -	0.024-
7.	Partial necrosis	0.055 +	0.168 +	0.002 –	0.019-
8.	Cowpea with local lesion	0.368 +	0.476 +	0.002 -	0.020-
9.	Healthy cowpea	0.010 -	0.009 -	0.007 -	0.015-
10.	Healthy sunflower	0.005 -	0.011 -	0.003 -	0.020 -

* Mean of three replications
+: Presence of virus
-: Absence of virus

Determination of titre value of polyclonal antiserum raised against sunflower necrosis virus

The titre value of SFNV antiserum was determined using DAC-ELISA as 1:200 where the maximum absorbance of 0.869 was recorded as against 0.060 in buffer control. The antiserum could be diluted upto 1: 2000 and used for the detection of antigen present in 1:100 dilution (Table 1).

Immunosorbent Electron Microscopy (ISEM)

Purified preparations of the virus negatively stained with uranyl acetate (2%; pH 4.5) was examined under transmission Electron Microscope (JEOL-100-CF II) that revealed the presence of isometric particles. Then they were trapped and decorated with the polyclonal antiserum raised against the SFNV.

Fagbenle and Ford (1970) reported that spherical virus particles were observed in a negatively stained preparation and with a diameter of 28-32 nm. Gracia and Feldman (1974) described that the TSV particles in pepper were isometric in shape and 32.8 (±1.9) nm in diameter. Leaf dip preparations of *C. quinoa* showed spherical virus particle and the diameter was 27 nm (Bos *et al*., 1980). Cook *et al.* (1999) observed the TSV in groundnut as spherical particles having a diameter of 30 nm when it was stained with 2 per cent uranyl acetate.

Detection of SFNV in infected samples using Direct Antigen Coating ELISA (DAC-ELISA)

Samples showing different symptoms on sunflower were initially tested for the presence of virus using polyclonal antiserum raised against SFNV and GBNV. None of the samples were positive to GBNV (TOSPO) whereas four samples exhibiting typical symptoms of SFNV *viz.,* complete stem necrosis, top necrosis, partial stem necrosis and mild mosaic were found to be positive for SFNV (Ilar) by recording the absorbance values of 0.254, 0.234, 0.055 and 0.040 respectively as against 0.005 in healthy sample after 1 h incubation (Table 2). The polyclonal antiserum raised against the isolate (92 / 475 of tobacco streak virus) infecting

Table 3. Detection of SFNV in infected samples using TSV (Groundnut) antiserum

Sample No.	Description	*Absorbance at 405 nm**	
		After 1 h	**After 2 h**
1.	Complete necrosis on stem, leaves and petiole of sunflower	0.061 +	0.108 +
2.	Severe mosaic	0.003 -	0.010 -
3.	Top necrosis	0.043 +	0.072 +
4.	Yellow blotch	0.004 -	0.015 -
5.	Mild mosaic	0.001 -	0.032 -
6.	Mild mosaic + yellowing	0.002 -	0.014 -
7.	Partial necrosis	0.001 -	0.021 -
8.	Cowpea with local lesion	0.067 +	0.192 +
9.	Healthy cowpea	0.005 -	0.017 -
10.	Healthy sunflower	0.002 -	0.028 -

***** Mean of three replications
+ Presence of virus
-Absence of virus

groundnut in South Africa was tested against sunflower samples. But, the reaction was very weak when tested with the infected samples. The presence of virus was detected only in two samples such as complete stem necrosis and top necrosis where the absorbance value was detected as 0.061 and 0.043 respectively as against 0.002 in healthy control after 1 h incubation (Table 3).

Prasada Rao *et al*. (2000) also proved by ELISA tests that TSV – sunflower was not reacting with PBNV (Peanut bud necrosis virus) and TSWV (Tomato spotted wilt virus) antiserum. Ramiah *et al*. (2001a,b) reported that TSV from sunflower failed to react with CMV (Cucumber mosaic virus) GBNV (Groundnut bud necrosis virus) and PVY (Potato virus Y) in DAC-ELISA. Reddy *et al*. (2002) observed that there was no reaction in DAC-ELISA when antisera of CMV-B, CMV-C and CMV-S isolates were used against TSV- groundnut but there was reaction with the TSV antisera. Salazer *et al*. (1981) reported that SB-10, a new strain of TSV was identified from potato by using ELISA. Heterologous antiserum raised against TSV-groundnut was found to be very weak when tested with the infected samples. Ilarviruses are generally known to be weak immunogens and TSV antisera raised to certain isolates often do not detect other isolates (Kaiser *et al*., 1991). Conversi (1972) proved that TSV-black raspberry isolate was different from TSV-red node strain of beans by ELISA.

Table 4. Distribution and concentration of virus in different parts of infected sunflower

Sl. No.	Parts of the infected plant	Absorbance at 405 nm*
1.	Leaf	0.719+
2.	Petiole	0. 501+
3.	Stem	0.893+
4.	Calyx	0.419+
5.	Pollen	0.141-
6.	Seed	0.124-
7.	Healthy leaf	0.106-
8.	Buffer	0.072

* Mean of six replications
\+ Presence of virus
\- Absence of virus

Table 5. Detection of SFNV through DAC- ELISA at different periods of inoculation

Sl. No.	Days after inoculation	Absorbance at 405 nm *	Symptom development
1.	3	0.134 -	Nil
2.	7	0.382 +	Development of necrotic spots on leaves
3.	10	0.523 +	One side of leaf lamina become necrotic
4.	14	0.851 +	Necrosis extends to petiole
5.	18	1.032 +	Part of the stem become necrotic
6.	21	1.060 +	Necrosis extends to bottom of the plant causing complete stem necrosis
7.	Healthy control	0.124 -	-
8.	Blank	0.011 -	-

* Mean of six replications
\+ Presence of the virus
\- Absence of the virus

Concentration of virus in different parts of infected sunflower

Different parts *viz.,* leaf, petiole, stem, calyx, pollen and seed were tested for the concentration at which the virus present in these samples by using DAC-ELISA. Concentration was found to be more in the stem portion that recorded the absorbance value of 0.893 which was followed by leaf (0.719), petiole (0.501) and calyx (0.419) whereas it was 0.072 in buffer control. The presence of SFNV was not detected in seed and pollen samples (Table 4).

Detection of SFNV through DAC-ELISA at different periods of inoculation

Twenty days old sunflower plants (Co 4) inoculated with SFNV were observed periodically for the symptom development. After seven days of inoculation, some necrotic spots started appearing on inoculated leaves. As the day progresses, one side of leaf lamina became necrotic (10 DAI) and the necrosis extended further to petiole (14 DAI) and stem (18 to 21 DAI) led to the death of the plant. Concentration of the virus also got increased with increase in days after inoculation. An absorbance value of 0.523 recorded at 10 days after inoculation was increased to 1.060 in 21 days after inoculation (Table 5).

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